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Chromosomal localization and characterization of c-*abl* in the t(6;9) of acute nonlymphocytic leukemia

(gene mapping/chromosomal translocation/chronic myelogenous leukemia/tyrosine kinase)

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ABSTRACT **Acute nonlymphocytic leukemia associated with the chromosomal translocation t(6;9)(p23;q34) is an entity that is frequently associated with basophilia, which it shares with chronic myelogenous leukemia. The breakpoint on chromosome 9, q34, appears to be cytogenetically identical in both malignancies and is the site of the cellular oncogene c-*abl*. We investigated the role of c-*abl* in cells from two patients with the t(6;9) using *in situ* chromosomal hybridization, Southern hybridization, and *in vitro* phosphorylation. We showed that c-*abl* is not translocated from chromosome 9, resulting in a breakpoint that is on the 3′ side of this gene. The t(6;9) translocation does not appear to result in the production of an aberrantly sized protein product or in the acquisition of *in vitro* tyrosine kinase activity. This is in direct contrast to the findings in chronic myelogenous leukemia, in which c-*abl* is translocated, leading to the production of a structurally altered c-abl protein with activated tyrosine kinase. Lastly, we demonstrated that the cells of one patient contain sequences from chromosome 9 inserted at the junction of a reciprocal translocation between chromosomes 4 and 10 on the 4q+ chromosome. This insertion, which is at least 100 kilobase pairs in length, represents a duplication and translocation of the protein coding region of c-*abl*.**

Human leukemias and lymphomas are frequently associated with chromosomal aberrations (translocations, inversions, or deletions) that are specific for particular disease subtypes (1). A relatively restricted number of chromosomal regions participate in these types of rearrangements; this has led to the suggestion that genes important to the transformation of a normal cell into a malignant cell are located at these sites (2). Studies of some of these breakpoints at the molecular level support this view. For example, the protooncogene c-*myc*, located on chromosome 8, participates in several possible translocations involving the immunoglobulin coding regions on chromosomes 2, 14, or 22 (3–5); activation of this gene appears to play a major role in the transformation process in Burkitt lymphoma.

One of the best characterized translocations is the t(9;22) in chronic myelogenous leukemia (CML), in which there is a reciprocal exchange of material involving the long arm of chromosome 9 and the long arm of chromosome 22 (6). In this process, the cellular oncogene c-*abl* is translocated from chromosome 9 to 22 (7), producing a chimeric gene that contains sequences from both chromosomes (8). This gene is then transcribed into a unique mRNA of 8 kilobase pairs (kbp), whereas the normal c-*abl* mRNA is 6 kbp (9, 10). The 8-kbp mRNA represents a fusion between the c-*abl* gene and the *bcr* gene on chromosome 22 (11) and gives rise to a protein with a molecular size of 210 kDa, which is 60 kDa larger than the normal cellular c-*abl* protein (12). Like the c-*abl* protein that is present in cells transformed by the Abelson-murine-leukemia virus, but unlike the normal c-*abl* homologue, this protein is capable of autophosphorylation of tyrosine (12). Thus, it seems likely that this translocation of c-*abl* activates the gene, ultimately leading to malignant transformation.

In view of the findings on c-*abl* in CML, it might be expected that other neoplastic diseases would also involve this gene. A possible candidate was the t(6;9) in acute nonlymphocytic leukemia (ANLL), originally described by Rowley and Potter (13) and later elaborated in detail (14, 15). This translocation involves a reciprocal exchange of genetic material between the short, or p, arm of chromosome 6 and the long, or q, arm of chromosome 9 [t(6;9)(p23;q34)]. Although these two types of leukemia, CML and ANLL, are clinically distinct, they have some features in common; both affect cells of the myeloid lineage, both are associated with marrow basophilia (15), both CML in blast crisis and ANLL are highly resistant to chemotherapy and, most importantly, the breakpoint on chromosome 9 appears to be identical at the cytologic level in both diseases. It seemed reasonable to hypothesize that at least some features of these two diseases are related to a common molecular mechanism.

We report here the investigation of the c-*abl* gene and its protein in malignant cells from two patients who had the t(6;9). In this study, we used *in situ* chromosomal hybridization, Southern hybridization, and *in vitro* protein phosphorylation. We show that, in contrast to the findings in CML, in ANLL with the t(6;9), the c-*abl* gene is not translocated from chromosome 9, the breakpoint does not lie within the gene, and an abnormal c-*abl* protein is not apparent. In addition, we demonstrate that, in one of the two patients, an extra copy of c-*abl* had been inserted at the junction of a reciprocal translocation involving chromosomes 4 and 10.

MATERIALS AND METHODS

Cytogenetic Analysis of Patient Samples. Since 1970, metaphase cell preparations adequate for cytogenetic analysis have been obtained at our institution from bone marrow or peripheral blood cells of 416 patients with ANLL and 250 patients with a myelodysplastic syndrome. Among these, five patients had malignant cells characterized by a t(6;9). We have described the clinical, morphologic, and cytogenetic features of these five patients and of four additional patients treated at other institutions (15). The malignant cells from two of these patients (patients 4 and 5 in ref. 15) were used for the present investigation.

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Abbreviations: ANLL, acute nonlymphocytic leukemia; CML, chronic myelogenous leukemia; kbp, kilobase pair(s).

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Cytogenetic analyses with trypsin–Giemsa or quinacrine fluorescence banding were performed on bone marrow aspirates obtained at the time of diagnosis (patient 1 and 2) and at clinical relapse (patient 2). Metaphase cells from patient 2 were examined from 24-hr unstimulated cultures; cells from patient 1 were methotrexate-synchronized and cultured for 48 hr in phytohemagglutinin-stimulated leukocyte-conditioned medium. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature (16). Abnormal clones were defined as two or more cells with the same structural rearrangement (13).

***In Situ* Chromosomal Hybridizations.** *In situ* chromosomal hybridization was performed as described (17). Radiolabeled probes were prepared by nick-translation of the entire plasmid with all four [^{3}H]deoxynucleotides to specific activities of 7.3×10^7 dpm/μg (v-abl), 9.1×10^7 dpm/ug (T-39-1), and 3.3×10^7 dpm/ug (T-39-2). Metaphase cells were hybridized with 20 or 40 ng of probe/ml of hybridization mixture. Autoradiographs were exposed for 11 days at 4°C.

Southern Hybridizations. High molecular weight DNA was prepared from patient leukocytes that were frozen or from human placenta as described (18). Samples of DNA were digested with restriction enzymes according to the manufacturer's recommendations. The samples were size fractionated by electrophoresis on 0.8% agarose gels and transferred to Gene-Screen Plus nylon transfer membranes (New England Nuclear) according to the manufacturer's recommendations. Hybridization mixes contained 50% (vol/vol) formamide, 5× SSC, 10% (wt/vol) dextran sulfate, 1% $NaDodSO_4$, 20 mM sodium phosphate (pH 6.8), 0.1% Ficoll, 0.1% polyvinylpyrrolidine, 0.1% bovine serum albumin, denatured salmon sperm DNA at 100 μg/ml, and 4×10^7 dpm of probe that had been nick-translated to a specific activity of $2–4 \times 10^8$ dpm/μg. Hybridizations were performed at 42°C for 16 hr, and the blots were then washed in 0.1X SSC/1.0% $NaDodSO_4$ at 65°C for 2–4 hr, except for v-abl probes that were washed at 50°C.

***In Vitro* Phosphorylation Reaction.** The procedure of Konopka *et al.* (12) was followed. Exponentially growing cell lines were freshly harvested, or viable patient cells that had been frozen in 10% (vol/vol) dimethyl sulfoxide in liquid nitrogen were rapidly thawed at 37°C. A total of 10^7 cells were suspended at 4°C in 5 ml of lysis buffer [1% Triton X-100, 0.1% $NaDodSO_4$, 0.01 M NaH_2PO_4 (pH 7.5), 0.1 M NaCl] with 5 mM EDTA and clarified by centrifugation at 100,000 × *g* for 1 hr at 4°C. Extract (1 ml) was immunoprecipitated with 5 μl of αpeX-5 abl-specific antiserum (12) and allowed to remain on ice for 24 hr unless otherwise specified. For mixing experiments, 0.5 ml of each of two extracts was combined and immunoprecipitated with 5 μl of antiserum/ml.

The proteins were collected on formalin-fixed *Staphylococcus aureus* (19), were washed twice with lysis buffer, once with 50 mM Tris, pH 7.5, and were then resuspended in 40 μl of 20 mM Pipes (piperazinediethanesulfonate), pH 7.0, 10 mM $MnCl_2$. Next, 5 μCi of ^{32}P-ATP (5000 Ci/mmole, Amersham) was added, and the mixture was incubated at 30°C for 5 min, after which the immune complexes were washed twice with lysis buffer and reprecipitated. The reaction products were analyzed by $NaDodSO_4$ polyacrylamide gel electrophoresis (20) and by autoradiography.

RESULTS

Chromosomal Analyses. Patient 1 was a 42-year-old man with a myelodysplastic syndrome (refractory anemia with excess blasts in transformation) which later evolved into overt ANLL (acute myelocytic leukemia with maturation). Basophils comprised 4.25% and 2.0% of bone marrow cells during the preleukemic phase and at the time of overt leukemia, respectively; the normal value is <0.2%. A t(6;9) was noted as the sole karyotypic abnormality in each of 20 metaphase cells examined at the time of diagnosis; his karyotype was 46,XY,t(6;9)(p23;q34).

Patient 2 was a 5-year-old child, also with acute myelocytic leukemia with maturation, whose bone marrow contained 12% basophils. Cytogenetic analysis revealed a t(6;9), as well as a reciprocal translocation involving the long arms of chromosomes 4 and 10, in 14 of the 15 metaphase cells examined. The karyotype was 46,XX(6%)/46,XX,t(6;9)(p23;q34), t(4;10)(q31;q22) (94%). These chromosomal rearrangements were also noted in all metaphase cells examined at the time of relapse. In eight of the nine patients reported on previously, a t(6;9) was the sole abnormality present (15); thus, patient 2 is the only one whose malignant cells had an additional rearrangement.

C-*abl* Remains on Chromosome 9. *In situ* chromosomal hybridization to metaphase cells prepared from leukemic bone marrow from patients 1 and 2 was performed with a v-abl specific probe, PAB1sub9 (21). The v-abl probe contains all of the viral sequences homologous to the human c-*abl* locus (Fig. 1). The results indicated specific labeling of the normal chromosome 9 homologue as well as the 9q+ chromosome (Fig. 2, Table 1). Of 100 metaphase cells examined from patient 1, 25 were labeled on the normal chromosome 9 or the 9q+, or on both of these chromosomes ($P < 0.0005$). Eleven labeled sites, 5.9% (11/187) of the total labeled sites were noted on the normal chromosome 9 at band q34. On the rearranged chromosome 9 (9q+), 10 labeled sites, 5.3% (10/187) were located at band q34 adjacent to the breakpoint. In contrast, 8 labeled sites were noted on the normal chromosome 6 (4.3%), and 8 grains were distributed along the 6p− chromosome (4.3%).

Similar results were obtained from hybridization of the v-abl probe to cells from patient 2 (Fig. 2). Twenty-five of the 100 metaphase cells examined were labeled on the chromosome 9 homologue. In particular, 13 labeled sites, 7.3% (13/179) were noted at band q34 of the normal homologue, and 12, 6.7% (12/179) were observed at band q34 of the 9q+ chromosome ($P < 0.0005$). The chromosome 6 homologues were not specifically labeled [normal, chromsome 6, 1.7% (3/179); 6p−, 3.4% (6/179)].

C-*abl* is Duplicated and Translocated in Patient 2. *In situ* hybridization of the v-abl probe to metaphase cells from patient 2 resulted in specific labeling of a third chromosomal region, in addition to band q34 of the normal and rearranged chromosome 9, namely, the breakpoint junction of the 4q+ chromosome, the chromosome 4 homologue that is involved in the t(4;10) (Fig. 3). Of the 100 metaphase cells examined, 17 were labeled on the 4q+ chromosome. Twelve grains, 6.9% (12/179) of the total labeled sites were clustered at band 4q31, the breakpoint in this reciprocal translocation. This labeling was equivalent to that noted on band q34 of the normal chromosome 9 homologue and on the 9q+ chromosome, representing 7.3% and 6.7% of all labeled sites, respectively (Fig. 2). The normal and rearranged chromosomes 10 were not specifically labeled.

Analysis of the 3′ Region of c-*abl* Fails to Reveal a Breakpoint. DNA from the two patients was digested with restriction enzymes *Bam*HI, *Hin*dIII, and *Kpn* I and analyzed by Southern hybridization to the probe PAB1sub9. This probe contains all of the v-*abl* sequences, and it hybridizes to a region spanning about 35 kbp in the human genome (Fig. 1). No new restriction fragments were seen (data not shown), suggesting that the gene was intact. This result is consistent with the results obtained by *in situ* hybridization.

Because the orientation of c-*abl* on chromosome 9 has been shown to be 5′ centromeric and 3′ telomeric (8), the breakpoint on chromosome 9 in the t(6;9) must lie at the 3′

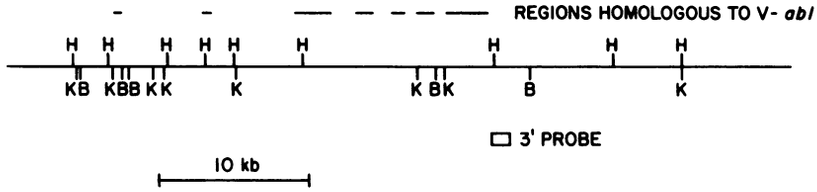

FIG. 1. Diagram of the human c-*abl* locus on chromosome 9. The lines show the v-*abl*-homologous regions. The open bar shows the subgenomic fragment, 28-1-2, used as a probe of the 3' sequences.

end of the gene. This region was investigated by Southern hybridization of genomic DNA of both patients with the probe 28-1-2, which lies immediately to the 3' side of the v-*abl* homologous sequences (Fig. 1). By using a combination of *Bam*HI and *Kpn* I digests, we found no new restriction fragments and were able to conclude that these sequences were intact (Fig. 4). The 3' end of the protein-coding region is within this area (11). Thus, the breakpoint on chromosome 9 must lie more than 18 kbp beyond the 3' end of the v-*abl* homologous sequences and beyond the last exon.

Investigation of c-*abl* Insertion into the 4q+ Chromosome in Patient 2. Insertion of c-*abl* into the junction of the t(4;10) requires that breakages occur at the 5' as well as at the 3' ends of the v-*abl*-homologous sequences. We investigated this by employing two genomic DNA probes, T-39-1 and T-39-2, that were cloned from the t(9;22) junctions from the malignant cells of two patients with CML. These sequences, which originate from chromosome 9 and lie 50–100 kbp to the 5' end of the v-*abl*-homologous sequences (unpublished observation), were used for *in situ* hybridization to metaphase chromosomes from patients 1 and 2 (Table 1). Consistent with the previous findings, both probes hybridized to the normal chromsome 9 and to the abnormal 9q+ chromosome in each patient; in addition, a significant number of grains was observed in patient 2 at the t(4;10) junction (Fig. 5), at the same location to which c-*abl* mapped (Fig. 3). *In situ* hybridization with the T-39-1 probe resulted in labeling of the 4q+ chromosome in 17/100 (17%) of the metaphase cells analyzed. Thirteen of the 185 labeled sites (7%) were noted at bands 4q31 and 10q22, the breakpoint junction of the 4q+ chromosome ($P < 0.005$). This labeling on the 4q+ chromosome was similar to that observed on the normal chromosome 9 or on the rearranged homologue (normal chromosome 9, 13 sites at 9q34; 9q+, 13 sites at 9q33 to 6pter).

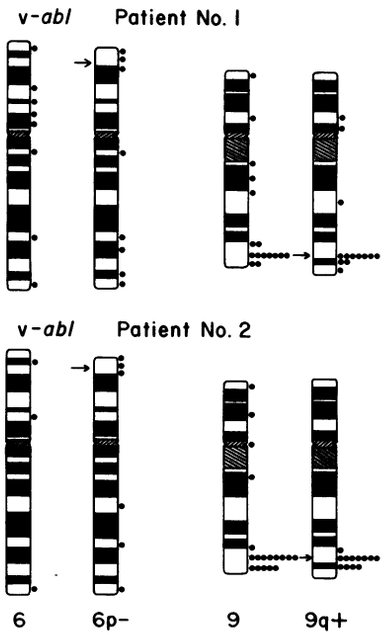

FIG. 2. Distribution of labeled sites on the normal chromosomes 6 and 9 and on the translocation derivatives 6p− and 9q+, in metaphase cells from two ANL patients with a t(6;9) that were hybridized to the v-abl probe PAB1sub9. One hundred metaphase cells were examined for each patient. The arrows identify the breakpoint junctions on the rearranged homologues. One dot represents one silver grain.

Of 100 metaphase cells examined after the hybridization with the T-39-2 probe, 21 were labeled on the 4q+ chromosome; 12 of these sites, 7% (12/173) were clustered at the breakpoint junction. The labeling of these sites is comparable to that observed on the normal chromosome 9, 6.4% (11/173) and on the 9q+ chromosome, 7.5% (13/173) at band q34.

Consistent with the above results, for both patients only germline restriction fragments were observed on Southern hybridization of this probe to DNA digested with *Bam*HI, *Xba* I, or *Bgl* II (probe T-39-1), and with *Bam*HI or *Hin*dIII (probe T-39-2). These results, combined with the above data on probe 28-1-2, show that the sequences from chromosome 9, which are translocated to the t(4;10) junction, are at least 100 kbp long. Based upon a map published by Shtivelman, this insertion contains the entire c-*abl* gene (11).

C-abl Protein in Cells With the t(6;9) Does Not Contain Tyrosine Kinase Activity. Viable cells from patient 1, which had been preserved in liquid nitrogen, were tested for *in vitro* tyrosine kinase activity. The BV 173 cell line was used as a positive control (22); this cell line was established from a patient with CML and contains an activated c-abl protein, p210, of molecular size 210 kDa (23). A negative control was provided by the Loukes cell line established from a patient with Burkitt lymphoma. As shown in Fig. 6, lanes 3 and 4, the t(6;9) cells did not contain abnormal tyrosine kinase activity; the pattern obtained was virtually identical to that obtained with the Loukes cells (lane 5). The lack of autophosphorylation was not due to proteolysis, as a mixture of extracts from the cells with the t(6;9) and BV 173 (lane 2) did not abolish the phosphorylation of p210 in the positive control (lane 1).

Metabolic labeling with ^{32}P and immune precipitation with specific antiserum revealed that the cells from patient 1 contained the normal p145 c-abl protein but did not have structurally altered c-abl protein homologues (O. Witte, personal communication). These data also confirmed that the cells were both viable and metabolically active.

DISCUSSION

We have shown in this study that the breakpoint on chromosome 9 in the t(6;9) in ANLL, although cytologically identical to that in CML, differs on the molecular level. The breakpoint in the t(6;9) is on the 3' side of the c-*abl* homologous sequences, whereas it is on the 5' side in CML. Furthermore, the c-abl protein in the malignant cells of the t(6;9) patient studied appears to be normal in size and *in vitro* to lack tyrosine kinase activity, whereas in CML the protein is abnormally large and has tyrosine kinase activity (12). Because only small amounts of patient material were available, we have been unable to determine whether the mRNA transcript is abnormal in size or in abundance in t(6;9) cells.

Table 1. *In situ* hybridization studies of metaphase cells with a t(6;9)

Probe	Patient*	Labeled sites, no.	Cells labeled on chromosome 9, no.	Labeled sites on whole chromosomes, no.†			
				Normal 6	6p−	Normal 9‡	9q+‡
v-abl	1	187	25	8(4.3%)	8(4.3%)	16(8.6%)	13 (7.0%)
	2	179	24	3(1.7%)	6(3.4%)	17(9.5%)	12 (6.7%)
T-39-1	1	184	28	8(4.3%)	7(3.8%)	16(8.7%)	16 (8.7%)
	2	185	27	2(1.1%)	6(3.2%)	17(9.2%)	14 (7.6%)
T-39-2	1	166	27	6(3.6%)	7(4.2%)	15(9.0%)	18(10.8%)
	2	173	30	5(2.9%)	5(2.9%)	16(9.2%)	16 (9.2%)

One hundred metaphase cells were examined for each probe from each patient.
*Patients 1 and 2 were previously reported as patients 4 and 5, respectively, in ref. 15.
†Values in parentheses refer to the percentage of total labeled sites.
‡χ^2 value corresponds to $P < 0.005$ for these chromosomes.

However, a change in the RNA transcript would seem unlikely, as the breakpoint must lie well beyond the 3′ end of the last exon.

Studies of the v-abl protein obtained from Abelson-murine-leukemia virus-infected cells have shown that the presence of tyrosine-specific kinase activity is required for transformation and that the addition of *gag* sequences at the amino terminus of this protein confer specificity (i.e., fibroblast vs. lymphoid transformation) (24). This also appears to be the case for a newly identified retrovirus, Hardy–Zuckerman-feline-sarcoma virus, which contains a transduced c-*abl* gene (25). Similarly, the addition of new sequences to the amino terminus of the c-abl protein in CML confers on it a tyrosine kinase activity that is remarkably similar to that seen in the viral protein (26). Thus, if the t(6;9) translocation activates c-abl, it does so in a manner that differs from its activation in CML or from that in the retrovirus induced tumors.

The identification of patients with "variant" translocations, involving exchanges between three or more chromosomes, can often provide insight into the problem of determining what is the critical chromosomal junction in a particular disease. Only one such variant related to the t(6;9) has been reported (27) and analysis of this translocation suggests that the consistent rearrangement involves the movement of material from 9q to 6p. Based upon that interpretation, the sequences necessary for the transformation would be created by the movement of sequences from the end of the chromosome 9, distal to c-*abl*, to chromosome 6, rather than by the addition of new sequences to the 3′ end of this gene.

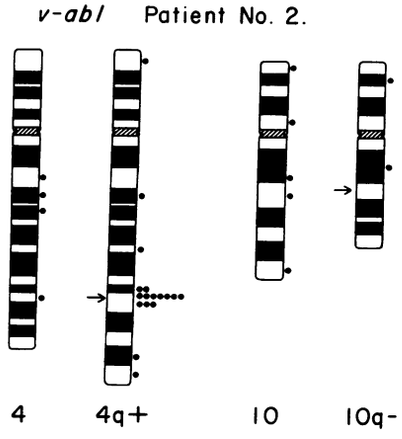

FIG. 3. Distribution of labeled sites on the normal chromosomes 4 and 10 and on the translocation derivatives 4q+ and 10q−, in metaphase cells from an ANLL patient (patient 2) with a t(4;10) (q31;q22) and a t(6;9); the cells were hybridized to the v-abl probe (PAB1sub9). The arrows identify the breakpoint junctions of the t(4;10). One dot represents one silver grain.

The additional copy of c-*abl* at the breakpoint junction of the t(4;10) in one of the patients was an unexpected finding. Since the t(4;10) was present at diagnosis and again at relapse, it is reasonable to assume that the abnormality confers a selective advantage to the patient's leukemic cells. We have shown that the duplicated region is at least 100–200 kbp in length, but smaller than the fraction of a chromosome band that can be observed on light microscopy, which is about 3000 kbp long. It is not possible at present to determine whether this duplicated region contains only sequences from chromosome 9 or, in addition, contains DNA from chromosome 6, thereby representing a duplication of the junction fragment.

It may be significant that patient 2 had 12% basophils in her bone marrow, a value that is 4- to 6-fold higher than that observed in any of the other eight patients with a t(6;9) in our series. This suggests that the "double dose" of c-*abl* or of nearby sequences was a contributing factor. It has been reported that infection of mouse bone marrow with the Abelson-murine-leukemia virus may, under certain conditions, give rise to basophilic leukemia (28); likewise, basophilia is commonly observed in CML. We have shown that c-*abl* on the 4q+ in patient 2 does not represent a hidden Philadelphia chromosome (that is, a 9;22 translocation), because *bcr* (29) was not rearranged on Southern hybridization of DNA from either patient 1 or patient 2 (unpublished results). Whether c-*abl* is directly involved in the basophilia and malignant transformation of these cells or is merely carried along as an innocent bystander on a relatively small

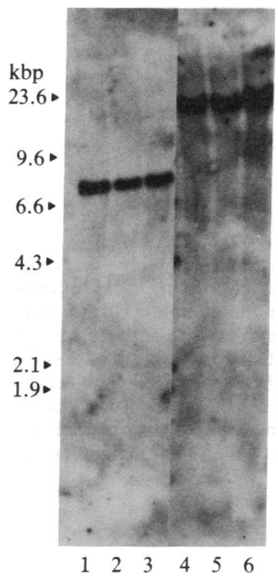

FIG. 4. Analysis of the 3′ end of the c-*abl* in t(6;9) cells. DNA (10 μg), isolated from cells of patients, was digested with *Bam*HI (lanes 1–3) or with *Kpn* I (lanes 4–6) and hybridized to probe 28-1-2. Lanes 1 and 4, patient 1; lanes 2 and 5, patient 2; lanes 3 and 6, normal human placenta.

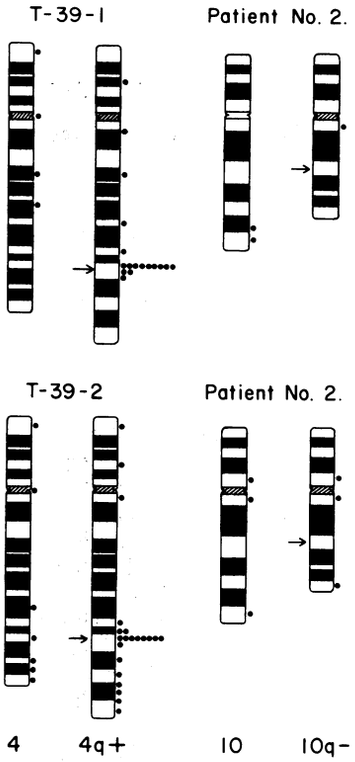

FIG. 5. Distribution of labeled sites on the normal chromosomes 4 and 10 and on the translocation derivatives 4q+ and 10q− in metaphase cells from patient 2 that were hybridized to the T-39-1 and T-39-2 probes. The arrows identify the breakpoint junctions of the t(4;10). One dot represents one silver grain.

piece of the genome, cannot be determined until this translocation is characterized further at the molecular level.

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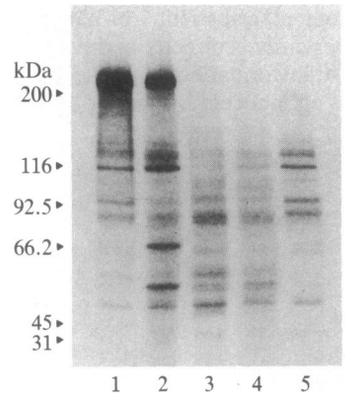

FIG. 6. *In vitro* phosphorylation of c-abl proteins. c-abl proteins were immunoprecipitated, and the immune complexes were incubated with [^{32}P]ATP. In lane 3, the incubation with antisera was for 2 hr; it was 24 hr in all other lanes. Each lane represents 2 × 10^6 cells. The immune complexes were electrophoresed in 10% polyacrylamide/NaDodSO$_4$ gels with molecular size markers as indicated. Lanes: 1, BV 173 cells; 2, BV 173 cells and cells from patient 1, 1-to-1 mix; 3, patient 1 (2-hr incubation); 4, patient 1 (24-hr incubation); 5, Loukes cell line.

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